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CONCEPTS AND SIGNIFICANCE OF ENZYME EVOLUTION

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It is evident from the papers presented at this meeting that the recent studies carried out in the field of comparative enzymology have stimulated an interest in the evolution of enzymes. I wish to summarize briefly some of the concepts and conclusions which appear to have developed during the course of these meetings. I will also present some of my own views on certain aspects of enzyme evolution.

Beginning in the late nineteen twenties, the interests of biochemists in comparative work were centered on wielding together the "unity hypothesis". This was a belief that biochemical activities of all organisms proceeded along generally similar lines. There is no question that this unity hypothesis has been confirmed and that it has become a basis for the notable advances in biochemistry as well as for the foundation of an understanding of mechanisms in enzyme evolution.

As has already been discussed in these meetings, it is reasonable to assume that the evolution of enzymes (or proteins) among organisms living today represents only

a minimal change compared to the vast changes that must have taken place before the occurrence of most primitive forms of life that now exist. In our discussions, we are not dealing with how the various types of enzymes arose but primarily with the modification during evolution of a given type.

Enzyme evolution, in general, has been ~~most~~ conservative. +/ very This has been necessarily so because there have been limitations to change owing to the restrictions imposed by the cofactors (coenzymes, metals, etc.). The vast number of comparative studies has clearly indicated that there has been practically no evolution of coenzymes. In my opinion, coenzyme alteration is not possible because a large number of enzymes require the same cofactor. In our studies with pyridine nucleotide analogues, we found that although some of the coenzyme analogues could replace the natural coenzyme with certain DPN-linked enzymes, other dehydrogenases were unable to react with the analogue. This is illustrated in the reaction of the acetylpyridine analogue of DPN with a series of rabbit dehydrogenases (Table I).[1] Hence, if 3-acetylpyridine were to replace nicotinamide as a vitamin, then a change in structure in all enzymes requiring DPN must be made to accommodate the acetylpyridine DPN. Since there are several hundred pyridine nucleotide-linked enzymes in most organisms, it would seem unlikely that all the enzymes could mutate in unison in order to be able to react with the new cofactor. Therefore, the evolution of dehydrogenases can proceed only to the extent that the mutated form can still react with its cofactor. Dr. Smith has already emphasized that even though considerable change has taken place in the cytochrome *c* obtained from different species, all cytochrome *c*'s still have the same unique oxidation-reduction potential, react with cytochrome oxidase, and are not autoxidizable.

We should also consider the relationships of enzymes that do not have prosthetic groups. This question is of interest, as indicated by the lively exchange of views between Dr. Watts and Dr. Smith with regard to the homology

TABLE I

Rates of Reaction of the 3-Acetylpyridine Analogue of DPN with Different Rabbit Dehydrogenases*

Dehydrogenase	Rate of AcPyDPN compared to DPN (%)
Liver alcohol	450
Liver glutamate	150
Heart mitrocondrial malate	125
Muscle lactate	22
Muscle D-glyceraldehyde-3-phosphate	10
Heart lactate	4
Liver β-hydroxybutyrate	< 1
Muscle α-glycerophosphate	0

* From N. O. Kaplan (1), p. 244.

of the subtilisins with the animal proteases. Whether all the proteolytic enzymes had a common origin is unanswerable at this time. The proteases are characterized by a diversity in specificities, and one might expect sequence differences in the enzymes examined. Furthermore, as Dr. Smith has pointed out, the subtilisins themselves show considerable variation in primary structure. Evolutionary changes in enzymes that have no cofactor requirements or with a broad specificity might be expected to occur at much faster rates than in enzymes that utilize cofactors and also in those which have definite substrate requirements essential for the maintenance of normal metabolic activities. In this connection, it is noteworthy that egg ovalbumin,[2] serum albumin,[3] and egg lysozyme[4] appear to be evolving at a much faster rate than intracellular enzymes.

The most reasonable way to show relationships among enzymes catalyzing the same function, as well as among enzymes within a given group (*i.e.*, the various dehydrogenases), would be to elucidate and compare the primary structures. The work on cytochrome *c* clearly shows the value of such an approach. To obtain meaningful data on evolutionary relationships, however, it is important to have the sequence of a large number of similar enzymes.

Conclusions drawn from sequence comparisons of five or six cytochrome c's are not so valid or even so interesting as when 25 or 30 primary structures have been determined. I believe that Dr. Smith has emphasized this point. One cannot draw conclusions ~~based on~~ an amino acid difference found in analyses of one member of a phylum, class, order, etc.; it is essential to examine a number of members before stressing differences.

As mentioned throughout this symposium, there are several million different living organisms and perhaps 100,000 different proteins in each organism. The purification and sequencing of large numbers of enzymes from different organisms seem logistically and financially impossible at this time, although improved automated techniques will certainly accelerate our rate of determination of primary structures of enzymes. Hence, we must rely on information from sequencing selected enzymes. Sequencing cannot be done with any precision on enzymes whose concentrations are low or where there is a scarcity of material.

Several methods have been used recently to show relationships among various groups of organisms. Catalytic comparisons sometimes yield data that are useful in showing these relationships. For example, the ruminants are the only group of mammals with a DPNase which is strongly inhibited by isonicotinic acid hydrazide.[5,6,7] This inhibition appears to be a characteristic of all members of the ruminant suborder, and indicates a subtle difference in structure of the DPNase as compared to other mammals. The DPN and TPN analogues can be used effectively to show that members of a given group have catalytically similar dehydrogenases. In fact, these catalytic comparisons at times can be used to show relationships of questionable or unclassified species. It seems better to classify the H or M lactate dehydrogenase (LDH) enzymes on the basis of their catalytic properties rather than their electrophoretic properties, since changes in physical properties are more apt to occur than changes in catalytic properties in closely related organisms.

In our studies, we have observed differences in

catalytic parameters of the same enzyme from different organisms. There is no doubt that, in general, during the course of the evolution of a given enzyme, catalytic changes have occurred. Cytochrome c, however, from different species seems to react identically with cytochrome oxidases from different sources. Hence, yeast cytochrome c reacts as well with the mammalian cytochrome oxidase as does mammalian cytochrome c. The cytochrome c example can be considered an exception, since it can be replaced as an acceptor by a number of dyes or even ferricyanide. The cytochrome can function as long as it has the correct redex potential and is autoxidizable. It will be of considerable interest to compare sequences of enzymes with different catalytic properties and specificities. I am biased toward comparative sequence studies of an enzyme where differences in catalytic properties have been shown to occur during evolution. From an evolutionary point of view such studies should prove to be valuable, but they also may be important in elucidating the intimate mechanism of action of the enzyme.

Physical measurements can be used to compare homologous enzymes. Studies carried out in our laboratory, primarily by Dr. Allan Wilson, have shown that heat stabilities of enzymes can be used as effective criteria to show the relationship of different groups of organisms. The H type LDH's in birds, as well as in the higher reptiles, are considerably more heat stable than those in other vertebrates. Electrophoretic migration of enzymes can be applied for comparative studies. Nearly all mammalian H type LDH's migrate very rapidly, and as such can be distinguished from the same type of enzyme in other vertebrates. In Table II are summarized the changes in heat stability and in electrophoretic migration of the H type LDH in the evolution of the vertebrates.[8]

It should be emphasized that conclusions such as drawn from the table cannot be made from analyses of a few members of a group but that a rather large sampling must be made. For example, approximately one hundred different mammalians have been investigated with respect to their H type LDH, and only a few have been found not to have the very

full page for table II

TABLE II

Temperature Stability and Electrophoretic Mobility of H_4 Lactate Dehydrogenase*

Taxonomic group	Inactivation temperature	Electrophoretic mobility
Neognathous Birds		
Passeriformes (6/67)	74	1.8
Piciformes (3/7)	79	2.8
Coraciiformes (2/9)	80	2.2
Trogoniformes (1/1)	68	0.2
Apodiformes (1/3)	77	2.2
Caprimulgiformes (1/5)	> 63	0.2
Strigiformes (1/2)	68	2.1
Cuculiformes (2/2)	80	2.4
Psittaciformes (1/1)	67	2.9
Columbiformes (2/2)	76	2.2
Charadriiformes (6/15)	77	1.5
Gruiformes (2/12)	63	2.2
Galliformes (1/3)	77	2.8
Falconiformes (3/5)	78	2.1
Anseriformes (1/2)	76	2.1
Ciconiiformes (1/7)	79	2.1
Pelecaniformes (3/5)	80	1.7
Procellariiformes (2/4)	76	1.5
Podicipediformes (1/1)	79	1.5
Gaviiformes (1/1)	80	1.8
Sphenisciformes (1/1)	79	2.0
Paleognathous Birds		
Tinamiformes (1/1)	80	6.6
Rheiformes (1/1) (Rhea)	79	6.8
Struthioniformes (1/1) (ostrich)	80	6.6
Higher Reptiles		
Caiman, *Caiman crocodilus*	76	5.1
Lizard, *Iguana iguana*	82	5.6
Lizard, *Varanus flavicens*	85	2.5
Snake, *Natrix sp.*	80	6.3
Snake, *Crotalus atrox*	80	6.3
Snake, *Constrictor constrictor*	77	5.3
Lower Reptiles		
Snapping turtle, *Chelydra serpentis*	58	6.1
Painted turtle, *Chrysemys picta*	52	5.7
Cooter turtle, *Pseudemys scripta*		6.3
Soft-shell turtle, *Trionyx ferox*	< 60	5.2

TABLE II (Cont'd.)

Taxonomic group	Inactivation temperature	Electrophoretic mobility
Mammals		
Man	65	15
Domestic cow	61	12
Domestic pig	61	16
Laboratory rabbit	65	12
Laboratory mouse	60	15
Laboratory rat	60	15
Squirrel, *Sciurus carolinensis*	68	15
European hedgehog, *Erinaceus europaeus*	65	13
Short-tail shrew, *Blarina brevicauda*	66	7.7
Opossum, *Didelphis virginiana*	60	15
Kangaroo, *Macropus robustus*	69	15
Amphibians		
Bullfrog, *Rana catesbiana*	52	10
Leopard frog, *Rana pipiens*	56	3.4
Toad, *Bufo marinus*	65	7.4
Congo eel, *Amphiuma tridactylum*	68	11
Bony Fish		
Sturgeon, *Acipenser transmontanus*	62	6.9
Haddock, *Melanogrammus aeglefinus*	63	7.8
Mackerel, *Scomber scrombrus*	60	6.1
Cartilaginous Fish		
Seven-gill shark, *Notohynchus maculatum*	68	5.0
Spiny dogfish, *Squalus acanthias*	64	4.1
Chimaera, *Hydrolagus collei*	< 65	7.0
Cyclostomes		
Lamprey, *Petromyzon marinus*		
Hagfish, *Eptatretus stouti*	< 65	4.8

* From N. O. Kaplan (1) pp. 268-269.

rapidly migrating enzyme. It is of interest that the exceptions have turned out to be in closely related species. One might expect to find some deviations in physical properties of an enzyme in a given group of organisms and in particular the electrophoretic parameters. However, if enough species are examined, I believe that an enzyme can be characterized for a given group (*i.e.*, class, order, suborder) by its physical properties. On the other hand, the exceptions may be interesting since they may represent

some unique change in primary structure that resulted in a distinct alteration of properties.[9]

Immunological methods can be applied to study relationships of an enzyme. We have used the microcomplement (C') fixation method developed by Wasserman and Levine[10] in our Department. This method has proven to be very useful in distinguishing small differences among homologous proteins. Compared to other immunological procedures, the micro C' fixation assay has considerably more resolving power, as illustrated in Table III.[8] With this sensitive method, Professor Levine and his associates have been able to distinguish single amino acid differences in hemoglobins.[11] Dr. Stanley Mills, at the University of California at San Diego, has been able by this

TABLE III

Comparison of Sensitivity of Immunological Methods*

Antiserum	Heterologous antigen	Cross reaction		
		Micro C' fixation	Macro C' fixation	Quantitative precipitin reaction
Anti-human hemoglobin A_1	Human hemoglobin S	41	86	100
Anti-human serum albumin	Chimpanzee serum albumin	52	97	89
Anti-chicken ovalbumin	Turkey ovalbumin	3	89	96
Anti-chicken H_4 LDH	Turkey H_4 LDH	32	112	91

* From N. O. Kaplan (1), p. 262.

method to recognize one amino acid change in the makeup of *Escherichia coli* tryptophan synthetase. The technique has been applied successfully to show the relationship of growth hormone[12] and serum albumin[4] in the primates. We have been able to show species relationship of a number of enzymes by the C' fixation method.[13] I have been impressed and I must say somewhat surprised by the good correlation obtained by this immunological enzyme approach and the known taxonomic classifications. An example of this

TABLE IV

Complement Fixation by Antiserum Against Chicken H Lactate Dehydrogenase*

Taxonomic group	Antiserum Amount Required for 50% C' F	Taxonomic group	Antiserum Amount Required for 50% C' F
Galliformes		Apodiformes	
Chicken	1.0	Swift	2.6
		Hummingbird	2.7
Falconiformes			
Hawk	1.5	Psittaciformes	
Sparrow hawk	1.5	Parakeet	2.8
Pelecaniformes		Strigiformes	
Snake bird	1.6	Owl	3.0
Procellariiformes		Ciconiiformes	
Wilson's petrel	1.7	Heron	3.0
Cuculiformes		Rheiformes	
Turaco	1.7	Rhea	3.1
Anseriformes		Podicipediformes	
Duck	1.7	Grebe	3.5
Goose	1.7		
Coraciiformes		Piciformes	
Kingfisher	2.0	Woodpecker	4.2
Columbiformes		Cuculiformes	
Pigeon	2.3	Cuckoo	5.7
Trogoniformes		Charadriiformes	
Quetzal	2.3	Woodcock	> 7.0
		Gull	8.0
		Murre	> 9.0
Gruiformes		Passeriformes	
Crane	2.4	Vireo	> 7.0
Rail	2.7	Sparrow	5.0
		Warbler	> 8.0

* From A. C. Wilson and N. O. Kaplan (13), p. 337.

correlation is given in Table IV, with the bird H type LDH. In this study, the antibody was directed against the crystalline chicken H LDH. As shown in the table, there is good agreement in the relative cross reaction among different species of the same order and also the cross reaction values are in accordance with the assumed taxonomic differences. It is essential to have a homologous enzyme for immunization in order to obtain meaningful data; if the antigen is pure, then the cross reactivity can be carried out in crude extracts; in this way it is feasible to compare

TABLE V

Micro Complement Fixation with Antisera to Pure Chicken Proteins*

Species	Antiserum concentration required for 50% C' Fixation					
	H_4 LDH	M_4 LDH	GPD	GDH	Aldolase	Hemoglobin
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Turkey	1.4	1.2	1.0	1.0	1.0	1.0
Duck	1.5	4.3	1.2	1.2		2.2
Pigeon	2.3	2.0	1.3	1.3		3.6
Ostrich	1.9	3.1	1.3	1.4	5.0	
Caiman	3.3	4.2	3.8	4.0	6.5	
Painted turtle	4.0	5.2	4.2	4.0		6.5
Bullfrog	14	40	30	19	18	
Sturgeon	80	20	12	25		
Halibut		> 200	> 50		> 100	
Dogfish	> 100	> 200	> 50			
Lamprey		30	> 50			
Hagfish	> 100	> 100				

* From N. O. Kaplan (1), p. 263.

homologous enzymes in a large number of species. In Table V the reactivity of a number of species against different chicken proteins is compared.

Certain precautions must be considered when immunological comparisons are made. For example, Dr. Morris Reichlin, formerly associated with Prof. Levine in our Department and now at the University of Buffalo, has been able to prepare antibodies against cytochrome *c*. He has found a relatively good correlation between cross reactivity using an antibody against horse cytochrome *c* and the taxonomic classification. However, in a few instances, marked inconsistencies have been found. Such inconsistencies have been emphasized in these meetings by Dr. Smith. Cytochrome *c* is quite a poor antigen and appears to have relatively few antigenic sites; hence when there is an amino acid substitution with considerably more antigenic potential and this change occurs in several unrelated species, this would tend to overshadow the effects of other antigenic sites and lead to rather similar immunological reactivities. In proteins such as LDH, glyceraldehyde-3-phosphate dehydrogenase (GPD), creatine kinase, etc., where there appear to be a multitude of antigenic sites, single amino acid changes do not have such a profound effect on the

micro C' fixation reaction. In general, in comparative studies with enzymes that are very antigenic, there has been an excellent agreement between relative cross reactivity by micro C' fixation and taxonomic distances.

I feel that the immunological method described above, when used properly, can be very useful in comparative studies. When more is known about the tertiary and quaternary structures as well as about the primary structure, we will be able to use the immunological methods to even better advantage. Before initiating sequencing on several homologous enzymes, it would be worth while to know whether they are immunologically distinct. Those that are immunologically distinct should have a primary structure which would actually make them more interesting than those with identical or quite similar immunological properties.

Based on the success with the abnormal hemoglobins, there has been a tendency in recent years to use "fingerprinting" as an indicator of relationships among homologous proteins. This approach can be valuable in comparative studies, particularly where large differences in primary sequence are involved; however, when small differences in structure are involved (*i.e.*, comparison of closely related species) invalid conclusions have been reached. Comparative "fingerprinting" can be misleading if there are impurities in the proteins studied or if the differences are not in changed groups.

There is also a danger in examining the sequence of one or two peptides of a given protein. This has already been indicated by Dr. Harris for the GPD. Table VI (from Dr. William Allison) is an extension of the data shown in Dr. Harris" presentation. If one examines the comparative sequences and knows nothing else about the enzyme, one might conclude that rabbit is more closely related to yeast than man. I wish to reemphasize what previous speakers have already stated—namely, that certain amino acid changes in a given enzyme may occur at random and therefore do not reflect the overall evolutionary characteristics of the protein. If one compares the immunological properties of the GPD's listed in Table V, very good agreement is found between the cross reactions and the taxonomic classifications.

TABLE VI

Amino Acid Sequences Around the Catalytically Active Sulfhydryl Group of D-Glyceraldehyde-3-Phosphate Dehydrogenase

Pig, Rabbit, Chicken, Ostrich, Sturgeon and Yeast
lys · ile · val · ser · asn · ala · ser · *cys · thr · thr · asn · cys · leu · ala · pro · leu · ala · lys
Man
ile · ile · ser · asn · ala · ser · *cys · thr · thr · asn · cys · leu · ala · pro · leu · ala · lys
Halibut
val · val · ser · asn · ala · ser · *cys · thr · thr · asn · cys · leu · ala · pro · leu · ala · lys
Lobster
asp · met · thr · val · val · ser · asn · ala · ser · *cys · thr · thr · asn · cys · leu · ala · pro · val · ala · lys

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The molecular weights of homologous enzymes have been discussed at great length at this conference. In our extensive comparative studies with the L specific LDH, we have found that the size of this enzyme is appreciably the same in all animals as well as in the few bacteria in which it has been studied. Since the molecular weights of the LDH's are roughly 140,000 and since there are four subunits, the primary polypeptide chain is about 35,000. These comparative studies suggest that there has been little change during evolution in the basic size of the structural gene for LDH. This appears to be true also for a diverse number of GPD's studied; again the subunit is approximately 35,000.

Malate dehydrogenases (MDH's) from a large number of animals (both mitochondrial and soluble forms) have been found to have molecular weights ranging from 60,000 to 70,000 [1, 14]. This would again indicate a subunit size of between 30,000 and 35,000. Reports on the plant MDH's give molecular weights approximating those found for the animal enzymes. This is also true for the *Neurospora* and yeast catalysts as well as for most bacteria. However, as found by Yoshida[15] as well as in our laboratory,[14,16] the *Bacillus subtilis* MDH appears to be about twice the weight of the other MDH's and consists of four subunits. Other members of the *Bacillus* group also possess the tetrameric enzyme; the larger MDH is also present in several other bacteria which may be related to the *Bacillus*. It is possible that the primary sequences of the *Bacillus* MDH's are of such a nature that they allow for the formation of a tetrameric structure. There appears to be no theoretical reason to restrict an active four-unit form when a dimeric structure is the predominant form found. However, a dimeric structure for the MDH has not been found. In fact, there is no convincing evidence as yet for the existence of an enzyme with an odd number of subunits.

It is interesting that the *Bacillus* enzyme can be dissociated into monomers by acid; on reneutralization the enzyme returns to its native four-unit structure. Although the Bacillus enzyme is larger, it does resemble the dimeric enzymes in some of its catalytic properties.

Dr. Tomkins has given a thorough discussion of the size and regulation of the beef liver glutamate dehydrogenase (GDH). Dr. Corman in our laboratory has been studying the dogfish GDH and details of his work have recently been published.[17] Unlike the beef liver dehydrogenase, the dogfish enzyme shows no aggregation characteristics. The molecular weight of the dogfish protein is 320,000, which is almost identical to that given by Dr. Tomkins for the unaggregated form of the beef enzyme. Although the dogfish dehydrogenase does not show the changes in molecular weight, it still responds to ADP and GTP in a fashion almost identical to that of the beef catalyst.[18] It thus appears that aggregation depends on a unique amino acid sequence, which may or may not be present in a homologous enzyme from different species. In the *subtilis* MDH there is no evidence that the enzyme at catalytic concentrations dissociates into a dimeric form.

Dr. Watts' comments with respect to the molecular weights of the guanidine kinases gave some perspective to the relationship of molecular weight to the evolutionary changes of a given enzyme. It seems reasonable to assume that the arginine kinase of invertebrates and the creatine kinases of vertebrates may be the result of a modification of a single gene. Although the two enzymes have different molecular weights (the arginine kinase has a single polypeptide and a molecular weight approximately one-half of that of the creatine kinases, which consists of two subunits), they appear to have many similar properties. Thus it would appear likely that subtle modifications of a gene may lead to a primary structure that can exist in varying degrees of polymerization. The finding of an arginine kinase with a dimeric structure emphasizes this point. I wish, however, to reemphasize the importance of specific primary sequences in determining the number of subunits in an enzyme molecule.

Perhaps one of the most intriguing questions in the study of enzyme evolution is whether changes in enzyme structure are of particular survival advantage to an organism. Does enzyme evolution reflect positive changes or

are the changes a result of random phenomena that are time dependent. It has been suggested, in particular for hemoglobin[19] and cytochrome c,[20] that mutational changes in proteins occur at a constant rate. This would imply changes without any specific physiological or survival implications.

I believe that enzyme changes as well as morphological changes follow the laws of natural selection. In this connection, it is of interest to note the function of multiple forms of enzymes. Prof. Wieland has already pointed out the possible difference in function of the two major types of LDH. Muscle aldolase is certainly better geared for breakdown of fructose 1-6 diphosphate, as compared to the liver enzyme. On the other hand, the liver aldolase seems more oriented to the formation of the fructose diphosphate. Hence, the liver enzyme appears to promote gluconeogenesis; whereas the muscle catalyst is so structured as to function more efficiently in glycolysis. The liver aldolase also is better able to cleave fructose 1-phosphate than the muscle enzyme is; this might be expected since the liver ~~can~~ convert fructose to glucose, whereas voluntary muscle does not.

Papadopoulos and Velick[21] have recently reported a second type of phosphoglyceraldehyde dehydrogenase in liver. This liver enzyme gives kinetics indicating greater ability to reduce 1, 3 diphosphoglyceric acid to the aldehyde, than is observed with the muscle phosphoglyceraldehyde dehydrogenase. As discussed above for the aldolase, the muscle dehydrogenase characteristically is directed toward promoting glycolysis. A number of glycolytic and related enzymes have now been found to exist in multiple forms, which have kinetic differences. These are listed in Table VII; the existence of such multiple forms appears to be related to their respective function in synthesis and breakdown of glycogen. Examination of the catalytic properties of mitochondrial and cytoplasmic forms of an enzyme shows differences that suggest functional variation between the two types, as with the MDH's and the transaminases. Multiple forms of an enzyme with functional differences certainly indicate phenomena of natural selection in enzyme evolution.

TABLE VII

Multiple Types of Glycolytic Enzymes

Lactate dehydrogenase
Creatine kinase
Aldolase (3)
Phosphoglucomutase
D-Glyceraldehyde-3-phosphate dehydrogenase
Glycerol phosphate dehydrogenase
Enolase
Hexokinase (Glucokinase)
Phosphofructokinase?
Phosphorylase

Enzyme duplication appears to be a much more widely occurring phenomenon than originally thought. By duplicate enzymes we mean multiple forms of enzymes with rather similar properties but different in their primary sequences and present in all members of one species. The mouse appears to have two duplicate M type LDH's, whereas, as far as can be detected, only one H form exists in the mouse.[22] The haddock also has two M types; we have been able to crystallize the two haddock M LDH's and, in preliminary studies, these M LDH's have been found to differ in only a few amino acids. The H and M types might also be considered "duplicates" and probably arose from gene duplication some time ago, but we know that the H and M LDH's are very different in their primary structures. We have restricted the term "enzyme duplication" to closely related enzymes such as the two M types; it is noteworthy that a number of lower vertebrates have duplicate H type LDH's. Table VIII lists the various forms of genetic variants.

"Allelic" enzymes can usually be distinguished from "duplicate" enzymes, because they are not present in all members of a given species and hence are structural variants of a single gene. Enzyme alleles are now quite a common phenomenon and a vast literature is beginning to build up in this particular area of enzymology. Dr. Stanley Salthe, who was formerly in our laboratory, has detected 15 distinct alleles of the H type LDH in different populations of *Rana pipiens*; however, as yet he has been able to detect only one variant of the M type enzyme in all the

TABLE VIII

Genetic Variants of Enzymes

A. Multiple Forms Due to Separate Genes and Forming Hybrids		
	Lactate dehydrogenase Creatine kinase Aldolase Phosphorylase	Catalytic differences significant Sequence differences large Immunologically distinct
B. Duplicate Enzymes. Closely Related Enzymes. Due to different genes but with only slight differences in sequence as well as in immunological, catalytic, and physical properties. Present in different levels in tissues of all animals of a species.		
C. Alleles of the Same Gene. Sequence differences small. But may have striking differences in physical and catalytic characteristics.		

populations that he has studied. The species which has so far been studied in most detail for polymorphism of enzymes is man. At least 20 different variants of glucose 6-phosphate dehydrogenase in human erythrocytes have been reported.[23, 24] Some of these variants not only show variation in their physical properties but may have very striking differences in their kinetic and specificity characteristics. The polymorphism of enzymes has opened new parameters of investigation for the geneticist, ecologist as well as the enzymologist.

The investigation of the functional significance of enzymes has drawn considerable new strength as the result of the great interest in allosteric enzymes. The fact that inherent within an enzyme is a control mechanism certainly points to the positive aspects of enzyme evolution. The purpose of this paper is not to dwell on allosteric enzymes; however, I wish to discuss briefly as an example of such a control mechanism the pyridine nucleotide transhydrogenase, which has been studied in our laboratory for a number of years. This enzyme, which has been purified from *Pseudomonas auriginosa*[25] and from beef heart,[26] catalyzes the following reaction

$$\text{TPNH} + \text{DPN} \longrightarrow \text{TPN} + \text{DPNH}$$

Although the oxidation-reduction potentials of the two pyridine nucleotides are almost identical, the reaction is not freely reversible. The reverse reaction with the *Pseudomonas* enzyme is practically negligible, except in the presence of 2' adenylic acid. The presence of this nucleotide allows the reaction to proceed toward equilibrium not only in the reverse direction but also in the forward direction given by the above equation.

With respect to the animal transhydrogenase, the oxidation of DPNH by TPN proceeds at about one-tenth the rate of the forward reaction. In the presence of ATP and mitochondrial particles (or subfractions), the rates of the reaction in the forward and reverse directions become approximately the same.[27,28] It is important to note that ATP does not have any effect on the purified animal enzyme. By control of the relative rates of reaction in the transhydrogenase reaction a mechanism is provided for regulating the levels of the reduced and oxidized forms of the pyridine coenzymes as well as for controlling the flow of electrons for either reductive synthesis or for obtaining useful energy in the form of ATP through the pathway of oxidative phosphorylation. It is now generally recognized that the potential energy inherent in DPNH is utilized for ATP formation through the electron transfer chain, whereas that of TPNH is for reductive synthesis. Hence, when there is an excess of TPNH, one might speculate that the excess could be drawn off by transfer to DPN and by so doing make possible the reducing potential of DPNH for synthesis of ATP. The fact that the reaction tends to go from left to right suggests that there is usually more TPNH generated than required for reductive synthetic reactions. However, at least with the animal system when there is an excess of ATP, there would be a backward flow to form TPNH, which would then be utilized in synthetic processes. In my opinion the transhydrogenase plays a vital role in balancing the rates of reductive synthesis and oxidative phosphorylation. The pyridine nucleotide transhydrogenase is only one illustration, of many illustrations, which shows that the catalytic properties of an enzyme may be very important physiologically. It will be of considerable interest eventually to understand how the regulatory mechanisms of an enzyme have evolved.

TABLE IX

Visual Pigments and Depths of Habitat of Marine Fishes*

Species	Summer Range of Depth (fathoms)	λ_{max} (mμ)
Summer flounder	2-10	503
Scup	1-20	498
Butterfish	1-30	499
Barracuda	1-10	498
Cod	5-75	496
Cusk	10-100	494
Lancet fish	> 200	480

* From Wald, Brown and Brown (29).

Perhaps one of the most interesting aspects is the correlation of differences in properties from different species of a given enzyme or protein with possible functional significance. For example, Wald and associates[29] have shown some years ago that the absorption maximae of rhodopsins from various salt water fish vary with the depth of the water in which the fish live. (Table IX). Hence, there appears to be an excellent correlation between the type of light available and the absorption maximae of the chromophoric group of the rhodopsins. Since the chromophore in all the rhodopsins is vitamin A_1 aldehyde, the changes are apparently caused by differences in sequences of the various opsins; these differences lead to structures that give alterations in the interaction between the protein and the vitamin A derivative leading to changes in the optical properties.

The nature of the enzymes in the thermophilic bacteria is also a subject of some interest. Do the proteins of these bacteria have unique properties different from those of the thermophilic bacteria? During the course of the comparative studies on MDH, Dr. William Murphey, in our laboratory, purified the enzyme from the thermophilic organism *Bacillus steareothermophilus*. This MDH like the enzyme from *B. subtilis* consists of four subunits. The *steareothermophilus* enzyme is considerably more heat

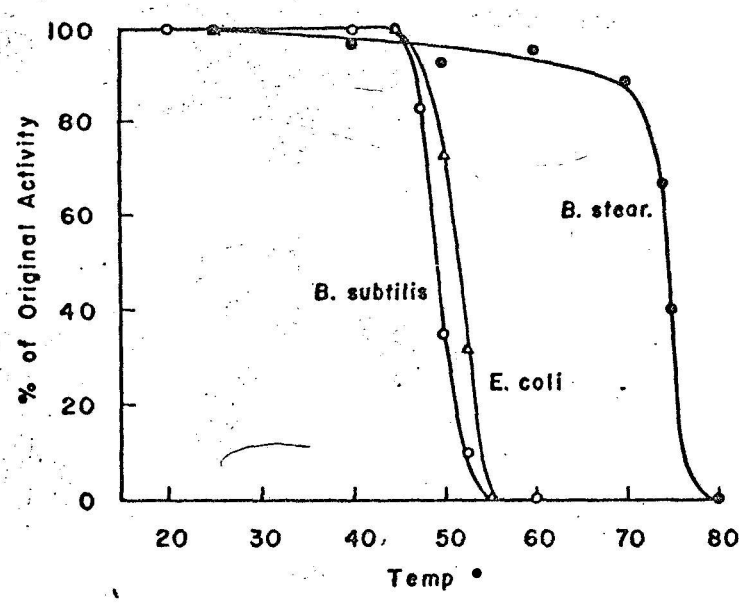

Fig. 1. Heat stability of purified MDH's from *B. subtilis* (○), *B. stearothermophilis* (●), and *E. coli* (Δ). The enzymes were diluted into isosatris buffer and incubated for 20 min at the indicated temperatures ± 0.5°; the initial rate of oxalacetate reduction was then assayed at room temperature. From Murphey *et al.*,[16] p. 1557.

resistant than the subtilis enzyme or the MDH from Escherichia coli[16] (Fig. 1). Furthermore, the thermophilus enzyme shows little catalytic activity at 30° and a maximal at 65°, which is distinct from the maximal activities of the other two organisms (Fig. 2). The *steareothermophilus* MDH is the same size and has other similarities to the *subtilis* MDH. In my opinion the unusual characteristics with respect to heat stability and activity result from a unique primary structure in the *thermophilus bacillus* enzyme and not from the interaction of small organic molecules or salts. It seems reasonable to assume that, as a whole, the enzymes from thermophilic organisms have structures that

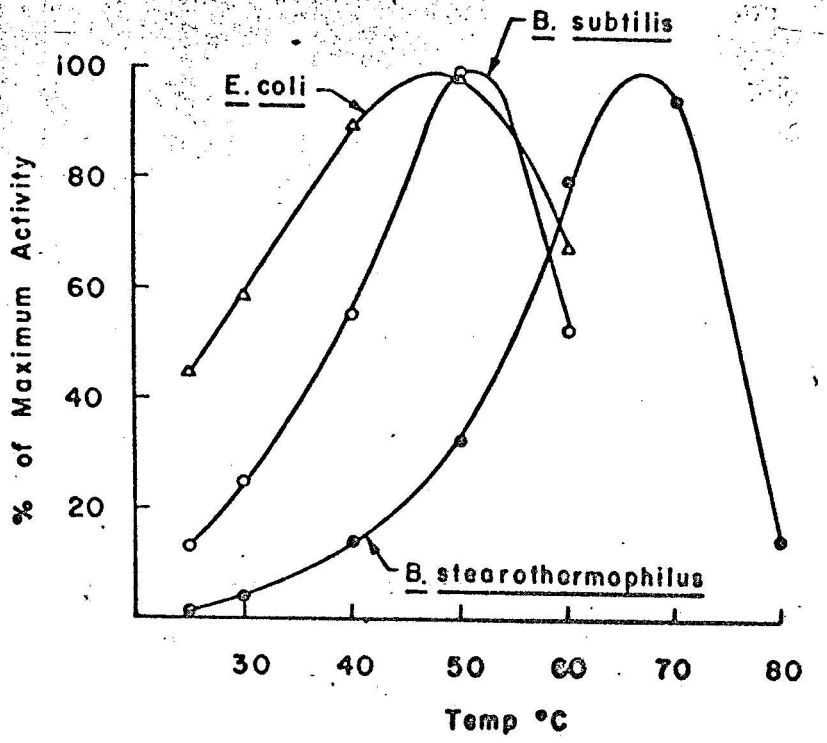

Fig. 2. Relative initial rates of oxalacetate reduction by MDH's from *B. subtilis* (o), *B. stearothermophilus* (●), and *E. coli* (Δ) as a function of the temperature of the assay mixture. Enzymes or dilutions thereof at room temperature were diluted 1:60 into the assay mixture which had previously been brought to the indicated temperature. From Murphey et al.,[16] p. 1557.

allow for increased heat stability and function at higher temperatures. In this connection, I wish to point out that fish living in cold water or the cold water lobster have LDH's geared to operate at maximal efficiency at low temperatures. The halibut LDH operates at 10° much like the mammalian or bird enzymes react at 37°.[1]

Another example of the relationship of the functional significance of enzyme evolution can be seen in the rabbit. This animal is known to have a high rise in blood lactate following muscular activity; in fact, it has been found to be able to maintain muscular contraction for a much longer

period under relatively anaerobic conditions than do most mammals. Inhibition by excess pyruvate of the rabbit M_4 LDH is not observed even when very large concentrations of the keto acid are used.[1] The rabbit M enzyme appears to be a much more effective pyruvate reductase than other mammalian M types that have been studied (*i.e.*, rat, mouse, beef, pig, and human). The characteristics of the rabbit enzyme, hence, can account for the elevated blood lactate in this species following exercise.

Before concluding, I shall discuss another aspect of comparative enzymology that is of particular interest. Dr. Harris has mentioned that the lobster GPD forms large crystals that have been found to be suitable for X-ray diffraction studies by Watson and associates;[30] a number of crystals of the same enzyme from other species have not been so satisfactory for these studies. Dr. Watson and his associates, in the Molecular Biology Laboratory at Cambridge, spent considerable time unsuccessfully attempting to grow large crystals of the enzyme from mammalian sources. Over the past few years in our laboratory, we have crystallized over 30 different animal LDH's. Only one of these, the dogfish M_4,[31] has been found to form extremely large crystals. Rossmann and associates[32] have found these crystals very appropriate for X-ray analysis; progress is being made by the Rossmann group on the study of X-ray structure, and we are now elucidating the total sequence of the dogfish enzyme. It is also interesting that the whale myoglobin turned out to be ideal for the X-ray solution of this protein.

By comparing the same enzyme from different sources, it is possible to study a specific catalytic or a physical characteristic of an enzyme. For example, with respect to the LDH's, we were first able to locate the essential sulfhydryl peptide involved in the binding of the coenzyme because the frog M type enzyme has a small number of cysteines, as compared to other LDH's.[33] In many instances an enzyme from one species has been found to be particularly suitable for purification, because of unusual stability. Different stabilities also allow for the selection of an appropriate enzyme for chemical modification.

Scrutiny of the properties of a number of enzymes catalyzing the same function allows for a much better understanding of the characteristics of an enzyme.

The great progress made in enzymology over the past few years allows for a much broader approach to the study of biochemical evolution. The papers presented and the comments generated during these meetings are strong indicators that the study of enzyme evolution will, in the future, be an important area of biological research. Furthermore, comparative studies appear to be important in elucidating the structural-functional activities of enzymes.

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